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Phosphopantetheinyl Transferase Catalyzed Site-Specific Protein Labeling with ADP Conjugated Chemical Probes

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Protein posttranslational modification (PTM) plays an important role in expanding the structural and functional diversities of proteins in the cell.[1] Among them, protein phosphopantetheinylation installs a 20 Å long phosphopantetheinyl (Ppant) group derived from coenzyme A (CoA) **1** on a conserved Ser residue in the acyl carrier proteins (ACP) of fatty acid synthase and polyketide synthase (PKS), and peptidyl carrier proteins (PCP) of nonribosomal peptide synthetase (NRPS). [2] The Ppant modification activates the carrier proteins for the attachment of biosynthetic intermediates during the enzymatic assembly of primary and secondary metabolites such as fatty acids, polyketides, and nonribosomal peptides (Figure 1A). In *Bacillus subtilis*, PCP modification by Ppant is catalyzed by Sfp phosphopantetheinyl transferase (PPTase), which shows significant substrate promiscuity with the chemical functionalities linked to the free thiol of CoA.[2b,3] Based on this feature, Sfp has been used for site-specific protein labeling by transferring synthetic probes such as biotin, peptide, fluorophore, and quantum dot from their CoA conjugates to PCP or short peptide tags fused to the target proteins.[4] Small molecule probes can be covalently attached to the free thiol at the end of the CoA Ppant arm through the formation of thioether, thioester, or a disulfide bond.[4] Synthetic strategies have also been developed to conjugate probes to the β-alanine or pantoic acid moiety of CoA.[5] We rationalized that a structurally simplified linker other than the Ppant group in the CoA−probe conjugate would further expand the scope of the small molecule probes for PPTase catalyzed protein labeling and facilitate the synthetic access to these probes as CoA analogues. However one concern for using CoA analogues with a nonnative Ppant linker as the substrate of Sfp is that the activity of the enzyme would be sensitive to structural variations in Ppant.[6] In this report we demonstrated that a mutant of Sfp, R4-4, catalyzes PCP labeling by chemical probes directly conjugated to adenosine 5′-diphosphophate (ADP) via a synthetic linker. The preparation of ADP conjugated probes can be accomplished with simple coupling reactions accommodating linkers of diverse structures between the probes and ADP. We envision the development of ADP conjugated small molecule probes for enzymatic protein labeling would allow the modification of target proteins with diverse chemical structures to manipulate their biological activities.

Recently we used a phage display to identify a mutant of Sfp, R4-4, which has three mutations, Lys28Glu, Thr44Glu, and Cys77Tyr, and catalyzes PCP modification with 3′-dephospho CoA (dpCoA) at a rate more than 300-fold higher than wild type Sfp.[7] R4-4 also catalyzes PCP modification with native CoA at a rate 10-fold higher than Sfp.[7] The high catalytic efficiency of R4-4 with both native CoA and dpCoA prompted us to test the activity of the enzyme with other CoA analogues. One class of CoA analogues we are interested in is ADP derivatives conjugated with small molecule probes through a structurally simplified linker other than the Ppant group in CoA (Figure 1B). The ADP−probe conjugate can be synthesized based on the coupling reaction between

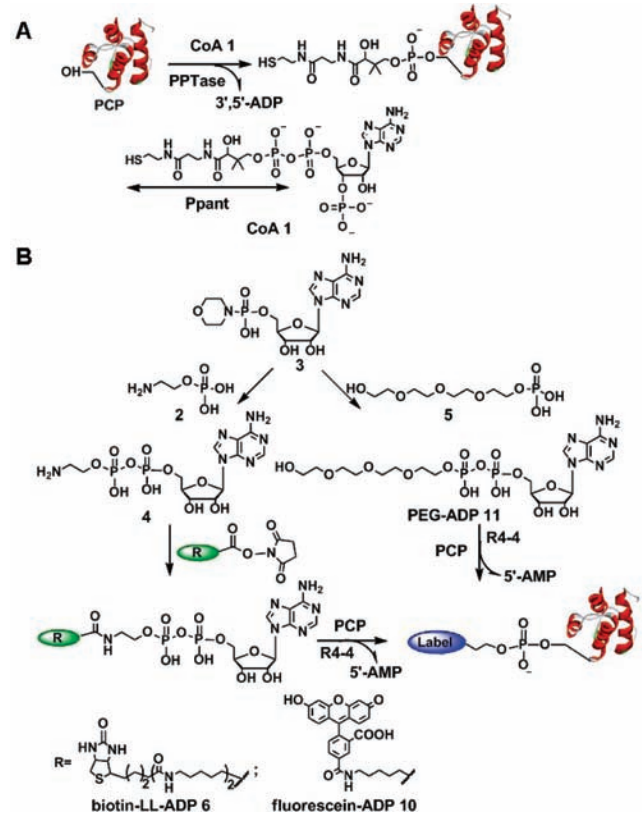

Figure 1. Site-specific protein labeling catalyzed by PPTases. (A) PPTase catalyzes covalent attachment of a Ppant prosthetic group to a Ser residue on PCP. (B) The design and synthesis of ADP conjugated chemical probes for PCP labeling catalyzed by Sfp mutant R4-4.

O-phosphorylethanolamine **2** and 4′-morpholine-*N*,*N*′-dicyclohexylcarboxamidinium salt (AMP-morpholidate) **3**[8] to install an amine functionalized linker on ADP (**4**) followed by the covalent attachment of small molecule probes through amide bond formation. Small molecules can also be linked to ADP by direct coupling between a phosphate derivative **5** and **3** (Figure 1B).

We first synthesized ADP conjugated biotin with a simple alkyl amide linker instead of Ppant (biotin-LL-ADP **6**). When **6** was used for the modification of GrsA PCP[9] catalyzed by R4-4, the MALDI-TOF spectrum of the reaction mixture confirmed the formation of modified PCP with a molecular ion peak at *m*/*z* 11 928 corresponding to the mass of the biotin−PCP conjugate (Figure 2A). The observed mass of biotin labeled PCP is 576 Da higher than that of apo PCP (*m*/*z* 11 352), matching the mass for the attachment of the biotin alkylphosphoryl group (MW = 576). In contrast, wild type Sfp did not catalyze PCP modification with biotin-LL-ADP **6**. This result suggests that R4-4 can use ADP conjugated small molecule probes as the substrate and enzymatically install a

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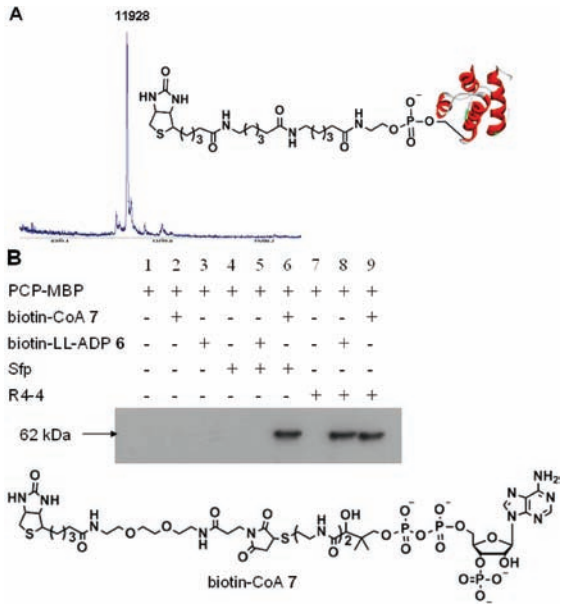

Figure 2. Site-specific protein labeling with biotin-LL-ADP **6**. (A) MALDI-TOF of PCP modified with **6** catalyzed by R4-4. (B) Western blot of PCP-MBP modified with **6** after probing with streptavidin-HRP.

structurally simplified linker replacing Ppant for the attachment of small molecule probes to PCP.

We then assayed the kinetics of R4-4 catalyzed PCP modification with **6** and compared the results with that of biotin-SS-CoA **8** and biotin-SS-dpCoA **9** as the substrates (Table S1, Supporting Information). **8** and **9** have biotin conjugated to native CoA and dpCoA through a disulfide bond at the end of the Ppant group.[7] The rate of R4-4 catalyzed PCP modification (k_{cat}/K_m) with **6** is ~300-fold less than that of **8** or **9**, denoting the importance of Ppant for enzyme catalysis. Nevertheless, R4-4 was able to achieve 100% PCP labeling with **6** within 1 h at 30 °C as shown in Figure 2A.

We also tested if PCP tagged fusion proteins can be labeled with **6** in the presence of R4-4. Fusion proteins were previously constructed with GrsA PCP fused to the N-terminus of maltose binding protein (MBP).[10a] The Western blot of the labeling reaction probed with a streptavidin-horseradish peroxidase (HRP) conjugate showed a band at 62 kDa (Figure 2B, lane 8), matching the size of PCP-MBP fusion. A band of the same size was shown in the labeling reaction with biotin-CoA **7** and Sfp. In contrast, no biotin labeled PCP-MBP fusion was observed when Sfp was used for protein labeling with **6** (Figure 2B, lane 5). Similarly, the ybbR tag, which is a short peptide substrate of Sfp,[10b] can also be labeled with **6** in the presence of R4-4 (Figure S1, Supporting Information). R4-4 catalyzed biotinylation of ybbR-MBP fusion was detected in the labeling reaction with **6** on the Western blot probed with streptavidin-HRP. These experiments demonstrate that chemical probes directly conjugated to ADP with a simple linker can be recognized as the substrates of R4-4 for protein labeling.

To test the scope of an R4-4 catalyzed protein labeling reaction, fluorescein and PEG groups were conjugated to ADP by a pyrophosphate linkage to afford fluorescein-ADP **10** and PEG-ADP **11** (Figure 1B). Labeling of PCP with **10** and **11** in the presence of R4-4 was confirmed by MALDI-TOF showing molecular ion peaks at *m*/*z* 11 952 for fluorescein labeled PCP and *m*/*z* 11 605 for PEG labeled PCP (Figure S2, Supporting Information). The increases in mass from apo PCP (*m*/*z* 11 352) match the conjugation of the fluorescein and PEG groups, respectively. We also used **10** to label PCP-MBP fusion catalyzed by R4-4. The labeling reaction mixture was analyzed by SDS-PAGE electrophoresis followed by fluorescence imaging (Figure S3, Supporting Information). R4-4 catalyzed protein labeling with **10** showed a fluorescence band of 62 kDa, matching the size of PCP-MBP fusion. However, wild type Sfp failed to catalyze protein labeling with **10**, demonstrating the expanded substrate specificity of R4-4 toward ADP conjugated small molecule probes.

In summary, we have developed a method to use Sfp mutant R4-4 for site-specific protein labeling with ADP conjugated small molecule probes. In this method, small molecules of diverse structures are directly conjugated to the 5′-diphosphate moiety of ADP and transferred by R4-4 to a specific Ser residue in the PCP or peptide tags fused to the target protein. Although the replacement of Ppant in CoA with structurally simplified linkers in ADP−probe conjugates results in lower catalytic activity of R4-4 for PCP modification, the labeling reaction can still achieve more than 90% yield of the modified protein within an hour of incubation. Small molecule probes can be linked to ADP by direct coupling of their phosphate derivatives to AMP-morpholidate **3**. A Ppant linkage in ADP−probe conjugates is no longer necessary for R4-4 catalysis. This would significantly simplify the synthesis of probe functionalized CoA analogues for PPTase catalyzed protein labeling. Furthermore, the catalytic activity of R4-4 with ADP conjugated probes would allow linkers of versatile length and structures to be installed between the small molecule label and the target protein, adding another layer of diversity to protein modification by PPTase.

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Supporting Information Available: Supplementary figures, experimental procedures, and full experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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